## REMARKS

Claims 1-18 currently are pending. Claims 1-8, 11-13 and 15-18 have been withdrawn from consideration by the Examiner. Claims 9, 10, and 14 currently have been amended.

**Restriction Requirement**

The Examiner made the restriction requirement final because the Examiner believes the technical feature claimed is not a contribution over the cited prior art.

Applicant respectfully request reconsideration of the Examiner's position. Although the Examiner cites some homologies after database searches, the Examiner has not put forth any anticipation or obviousness rejections of the claimed sequences. This indicates that the claimed sequences are novel, nonobvious and represent a contribution over the prior art according to PCT Rule 13.1 and 13.2. Therefore, applicants respectfully request that the Examiner withdraw the restriction requirement and examine all of the claims together.

**Sequence Listing**

The Examiner stated that in order to comply with the sequence rules applicants must identify sequences by providing SEQ ID NO: because the amino acid sequences presented on pages 17-19 do not have SEQ ID NOs.

In response, applicants herein submit the sequence listing again (first submitted on September 11, 2002) in paper form and on CD (computer readable form). The content of the paper copy of the Sequence Listing and the copy of the Sequence Listing in computer readable form is the same, and includes no new matter.

Presumably, the sequences to which the Examiner refers on pages 17-19 are the primers designated by applicants as SEQ ID NOs 6, 7, 8 and 9. The specification has been amended to add SEQ ID NOs where these primers appear.

**Claim Objections**

Claims 9, 10 and 14 are objected to under 37 CFR 1.75(c), as being of improper dependent form for failing to further limit the subject matter of a previous claim. The Examiner stated that claims 9 and 10 directly or indirectly depend upon non-elected claim 1.

Applicants do not believe claims 9, 10 and 14 are directed to non-elected subject matter. According to the Examiner's restriction requirement dated September 28, 2004, the Examiner stated that Group V encompasses claims 9, 10 and 14. Claims 9, 10 and 14's dependence on claim 1 does not necessarily indicate that they are now directed to non-elected subject matter. Claims 9, 10 and 14 are drawn to the use of a protein having dihydroorotase activity to identify an inhibitor and this protein is encoded by a DNA sequence as claimed in claim 1 or a DNA sequence having a homology of at least 40% with respect to SEQ ID NO: 1.

Applicants herein amend claims 9 and 10 as the Examiner suggests under section 6(b) of the Office action dated February 25, 2005. Applicants believe claim 14 is clear as it stands. Therefore, claim 14 has not been amended.

**35 USC § 112, ¶1**

The Examiner rejected claim 9, 10 and 14 under 35 USC § 112, ¶1 as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession

of the claimed invention. The Examiner believes the specification only provides a single representative species of dihydroorotase from *Solanum tuberosum* for use in the method of screening for herbicidally active test compounds, wherein the inhibition of the dihydroorotase activity is taken as a measure of the effectiveness of the test compounds.

The Examiner rejected claims 9, 10 and 14 under 35 USC § 112, ¶1, because the specification, while being enabling for a method (or process) for finding herbicidally active substances by inhibiting the activity of a plant dihydroorotase, comprising producing dihydroorotase recombinantly using the DNA sequence of SEQ ID NO: 1, does not reasonably provide enablement for using any DNA sequence having at least 40% homology to SEQ ID NO: 1 and which encodes a protein having the biological activity of a dihydroorotase.

The Examiner believes the instant specification gives no examples that dihydroorotase (DHO) is suitable as a target for herbicides and asks for data regarding herbicidal compounds acting on DHO.

In response, applicants point out that in addition to SEQ ID NO: 1 from *S. tuberosum*, on page 2, line 9 DHO from *A. thaliana* is disclosed and this can be used according to the present invention. One of ordinary skill in the art easily would be able to find other DHO sequences, for example from other plant species based on sequence similarity or mutagenesis techniques.

Also, functionally unrelated DNA would not fall under the scope of present claim 9. Multienzyme DHO complexes such as those from yeast or *D. melanogaster* also would not be within tthe scope of claim 9 as they are not plants.

DHO clearly is identified as an herbicide target. In the present examples, antisense

expression of functional DHO is done and the analysis of these transgenic plants shows that the reduction of DHO is correlated with growth retardation (Example 7).

Applicants also do not agree that the pending claims are directed to any DHO of certain homology. Claim 9 is directed to plant DHO, not any DHO. Also claim 9 herein has been amended to 60% identity instead of 40% identity which further restricts the sequences covered by the claims.

Applicants believe that screening for mutant DHO would be routine for one of ordinary skill in the art and can be done for example, by *in vivo* mutagenesis. This method would use *E. coli* strains having mutations in the genes for DNA repair system (e.g. mutHLS, mutD, mutt, etc.see Ruppl W.D. (1996), reference attached.) One of ordinary skill in the art would not have to undergo undue experimentation to obtain the modified DHO sequence. The use of these sequences is illustrated in Geener et al. (1994) and it is attached to the present response.

In sum, applicants respectfully request that the Examiner withdraw the rejections under 35 USC § 112, ¶1, because in the claims, the function is clearly assigned (DHO), the function/structure relationship clearly is established, there is disclosure of assays which can be used for identification of functional activity, the skilled artisan can screen other plant organisms to find naturally occurring variants of the sequences disclosed in the present application, and mutagenesis techniques for obtaining functional equivalents are within the level of the ordinary skilled artisan.

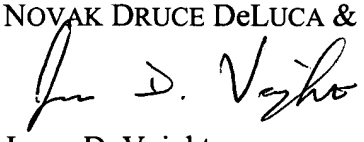
In view of the present amendment and remarks, applicants consider that the rejections of record have been obviated and respectfully solicit passage of the application to issue.

Applicants enclose the two month extension of time fee for $450.00 with form PTO-2038.

Please charge any shortage in fees due in connection with the filing of this paper, including Extension of Time fees to Deposit Account No. 14-1437. Please credit any excess fees to such deposit account.

Respectfully submitted,

NOVAK DRUCE DeLUCA & QUIGG, LLP



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# XL1-Red: A Highly Efficient Random Mutagenesis Strain

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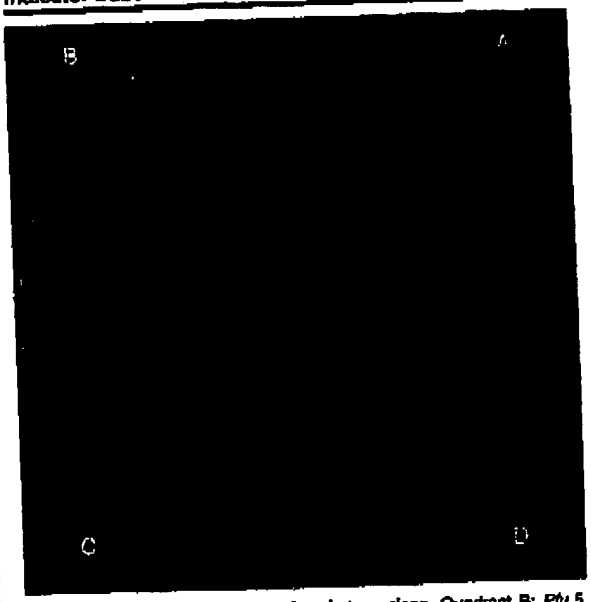
*Stratagene Cloning Systems*

*Cloning and sequencing a gene of interest are often only the first steps in understanding how the gene product functions, how it is regulated and what gene products it interacts with. To answer these complex questions, researchers traditionally generate mutations within the gene of interest and study their effects. While the structure and function of many genes are known, many others have not been characterized at the molecular level. The isolation of mutants, therefore, requires an approach that introduces random mutations throughout the gene. This technique has usually been reserved for genes that have a selectable or screenable phenotype in* Escherichia coli *because the isolation of random mutants is unpredictable and irreproducible. We have developed a highly efficient and reproducible method for isolating random mutations in a gene of interest using Stratagene's new XL1-Red mutator strain. This method does not require any genetic selection or screen for the gene's activity, nor does it require extensive genetic or biochemical manipulations.*

Over the years, there have been a number of procedures developed to generate random mutations within a gene. One method that is still widely used is the chemical treatment of DNA (see reference 1 for a review). Carried out *in vivo* or *in vitro*, this process uses nitrosoguanidine, hydroxylamine, ultraviolet light, etc., to generate the mutations. These chemical mutagens are usually employed when there is a genetic screen for the mutant phenotype because the efficiency of mutagenesis is difficult to control and monitor. The chemical method is rarely used on genes that have no screenable phenotype because the percentage of molecules having random changes is usually very low. In addition, treatment with different chemicals is not entirely random because mutational hot spots exist within the DNA, and only certain types of mutations (i.e., transitions or transversions) are generated depending on the chemical used (see table 4.2 in reference 1).

Recently, many laboratories have begun using the polymerase chain reaction (PCR)[2] to generate random mutations in genes that have no selectable phenotype. This method exploits the inherent infidelity of *Taq* DNA polymerase during the reaction. By varying the reaction conditions, the PCR process can yield random mutations, but there are two problems associated with this procedure. First, the gene of interest must be recloned into a vector after the PCR reaction. Second, because of the clonal expansion during the PCR amplification reaction, to guarantee that independent mutations have been generated, individual reactions should be performed. This makes the method very time-consuming and expensive, and only a limited number of random mutants can be generated.

***Figure 1***
***Mutants of the P. furiosus Alkaline Phosphatase Gene: Morphology of Clones on LB plates Containing BCIP Indicator Substrate***




Quadrant A: Parent *Pfu* alkaline phosphatase clone. Quadrant B: *Pfu* 5. Quadrant C: *Pfu* 5-1. Quadrant D: *Pfu* 5-2.

## Mutator Strains with High Random Mutation Rate

Stratagene has improved upon another method occasionally used for the generation of random mutants: the propagation of a clone in an *E. coli* mutator strain. Certain strains of *E. coli* that carry mutations in one of the DNA repair pathways are called mutator strains because their random mutation rate is higher than that of the wild-type parent. Typically, strains that carry the *mutS* or *mutD* allele have a 50- to 100-fold higher spontaneous mutation rate. Propagating a plasmid in one of these strains will eventually generate random mutations within the gene of interest; however, the increased mutation rate is still too low to be useful for genes that have no selectable/screenable phenotype. If the spontaneous mutation rate could be increased beyond the 50- to 100-fold higher level, then this easy and rapid technique could be exploited.

We have constructed an *E. coli* strain called XL1-Red, which is deficient in three of the primary DNA repair pathways in *E. coli*. Using basic bacterial genetics, we introduced the *mutS* (error-prone mismatch repair),[3] *mutD* (deficient in 3′ - 5′ exonuclease of DNA polymerase III)[4] and *mutT* (unable to hydrolyze 8-oxodGTP)[5] mutations into a single *E. coli* strain. The random mutation rate in this triple mutant strain was measured to be approximately 5000-fold higher than that of its nonmutant parent. Assuming that the spontaneous mutation rate in the wild-type *E. coli* is approximately $10^{-10}$ per nucleotide per generation[2] and that 30 generations are required for a single colony to reach stationary phase, every pBluescript® phagemid, pUC or other high-copy-number plasmid should carry one or more mutations in our XL1-Red triple mutant derivative.

Because the gene of interest represents only a portion of the plasmid length, we measured the spontaneous mutation frequency on a per base level as follows. XL1-Red was transformed with pGC10, which is derived from the pBluescript II phagemid and carries both ampicillin and chloramphenicol resistance. Ampicillin-resistant transformants of XL1-Red were pooled and grown to stationary phase, then their plasmid DNA was isolated by miniprep and retransformed into the XL1-Blue nonmutator strain. Ten clones were randomly selected, and a 1000-bp segment of each was subjected to sequence analysis. Because we selected for ampicillin resistance and a functional origin of replication, the region chosen for analysis overlapped the nonessential chloramphenicol-resistance gene.

### One Base Change per 2000 Nucleotides

The sequence data presented in table 1 show that, of the ten molecules sequenced, five contained no changes and five carried a single point mutation. Therefore, if the clone of interest is propagated in a pBluescript- or pUC-derived high-copy-number plasmid, overnight growth in the mutator strain will result in approximately one base change per 2000 nucleotides. If the gene of interest is 2000 bp in length, then on the average, every single isolate should have one point mutation. If the gene of interest is smaller, then either a percentage of isolates will be mutant (i.e., half of those 1000 bp in length), or by propagating the clone for additional rounds of growth, the mutation rate on a per base level will rise proportionally. In addition, if the gene of interest is cloned in a lower-copy-number plasmid (i.e., pBR322, pACYC177, etc.), the XL1-Red strain can still be used in the manner described, except that the clone should be propagated in the mutator strain for additional generations to account for the lower relative gene dosage of the target sequence.

The process of propagating a clone in the mutator strain makes it extremely easy and cost-effective to generate random mutants in a gene that does not have any selectable or screenable phenotype. In addition, although the sequence data are limited to the results presented, the types of mutations we have observed appear to be random, both in location and in the type of change. Of the five single mutations found (table 1), three were transition mutations, one was a transversion, and one was an insertion of one base.

### Protein Engineering Using XL1-Red Mutator Strain

The major advantage of performing random mutagenesis in XL1-Red is its efficacy when there is no selectable/screenable phenotype of the gene of interest. However, if there is a genetic screen to monitor mutations, the mutator strain makes it very easy to isolate mutants of interest. We have transformed the mutator strain with a pBluescript phagemid derivative that carries the alkaline phosphatase gene from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. The cloned gene expresses little alkaline phosphatase activity at 37°C, as judged by colony morphology (color) on LB plates containing the substrate BCIP, a chromogenic compound that will turn colonies blue when the substrate is dephosphorylated. Miniprep DNA isolated from an overnight culture of XL1-Red carrying the *P. furiosus* alkaline phosphatase clone was retransformed into XL1-Blue and plated on BCIP plates with ampicillin. Approximately 0.1% of the transformants exhibited a significantly darker blue phenotype. Miniprep analysis and retransformation of these mutants into nonmutator *E. coli* confirmed that the altered phenotype resulted from a plasmid mutation because all transformants exhibited the enhanced blue color characteristic of the mutants. Additionally, besides isolating variants that were more active at 37°C, we identified a significant population (1-2%) of colonies that were white, indicating that a mutation resulting in the loss of the basal alkaline phosphatase activity had occurred.

### First- and Second-Generation Mutants

One of the more highly active variants of the *P. furiosus* alkaline phosphatase gene was retransformed into XL1-Red and grown to stationary phase. Plasmid DNA was isolated and used to transform XL1-Blue again. Out of approximately 5000 transformants screened, two were observed to exhibit even greater alkaline phosphatase activity at 37°C than either the wild type or the original mutant. Miniprep analysis and retransformation confirmed that these second-generation mutants resulted from plasmid-borne change.

The parent *P. furiosus* alkaline phosphatase gene, the first-generation mutant (*Pfu* 5) and the second-generation

*Table 1*
***Sequence of a 1-kb Segment from pGC10***

| CLONE # | CHANGE(S) OBSERVED |
|---|---|
| 1 | 1-bp insertion at base 182 |
| 2 | G - A transition at base 149 |
| 3 | none |
| 4 | none |
| 5 | T - A transversion at base 42 |
| 6 | none |
| 7 | T - C transition at base 243 |
| 8 | G - A transition at base 103 |
| 9 | none |
| 10 | none |

Plasmid pGC10, a pBluescript phagemid derivative, was propagated for approximately 30 generations in XL1-Red. Its DNA was then isolated and retransformed into XL1-Blue. Ten clones were selected at random, and a 1-kb segment was sequenced.

mutants (*Pfu* 5-1 and *Pfu* 5-2) were then subjected to DNA sequence analysis. The sequence data confirmed that a mutation occurred within either the structural gene (*Pfu* 5-1 and 5-2; increase in activity at 37°C) or the vector (*Pfu* 5; enhanced expression at 37°C). Figure 1 shows the phenotype of the clones on plates containing the BCIP chromogenic substrate.

## Conclusion

Stratagene has developed an efficient, rapid and extremely easy method for introducing random mutations into a cloned gene of interest. This method simply requires transforming the cloned gene into our new XL1-Red mutator strain, whose random mutation rate is approximately 5000-fold higher than that of its wild-type parent. This strain is particularly suitable for generating random mutations within a gene that has no selectable or screenable phenotype. Additionally, when a genetic screen is available (such as for alkaline phosphatase), the use of the XL1-Red strain makes it extraordinarily easy to isolate variants with altered activity.

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5. Cox, E.C. (1976) *Ann. Rev. Genet.* 10: 135-156.


† The Polymerase Chain Reaction (PCR) process is covered by patents owned by Hoffmann-La Roche. Use of the PCR process requires a license.

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# Lab Tips

## Two Ways to Pour Perfect Sequencing Gels

Pouring a "good" gel can often be the most troublesome part of sequencing. To increase your chances of pouring that perfect gel, we recommend two easy methods. But first, here are a few suggestions about plate preparation. (1) Make sure the plates are as clean as possible. (2) Avoid using vacuum grease to seal the plate edges because it can be difficult to completely remove from the plates. (3) Try an alternative to the traditional but time-consuming process of using tape to seal the plate edges. Which brings us to the first of our two preferred methods for pouring gels quickly and painlessly.

### 1. Tapeless Method

This method is used for large plates such as the 37.5-cm-wide plates run on the Stratagene BaseAce® vertical sequencing apparatus. To prepare the plates, use a 1.5- to 2-cm-wide strip of Whatman® 3MM chromatography paper as a bottom spacer, then set up the side spacers as usual, and secure the sides and bottom of the gel with large binder clips. Set the prepared plates on Kimwipes® boxes or the polystyrene foam lids of your Stratagene packing boxes. Tilt the gel plates to a 45° angle from horizontal (top edges up) and pour the acrylamide slowly and carefully between the glass plates. Insert the comb and leave the gel to polymerize in a position 5° from horizontal, with the top of the gel slightly higher than the bottom. After pouring the gel, yet before the gel has polymerized, remove the clips from the bottom of the gel. The bottom spacer can be left in while running the gel because the current will run through it.

### 2. Hinge Method

You can use the hinge technique with the BaseAce Jr. vertical sequencing apparatus or any gel rig with plates approximately 22 cm wide. This method can be messy, so it is important to first lay down absorbent paper and be careful with the liquid acrylamide. Place the tall plate on blocks that are 5-12 cm high; polystyrene foam packing lids, empty tube holders or Kimwipes boxes all work well. Wet the spacers with water (this helps them adhere to the plate) and align them on the sides of the tall plate. Place the short plate on top, keeping the side and bottom edges even with each other. Put a piece of sequencing tape across only the bottom of the plates, so the tape acts as a hinge. Lift the short plate from the top edge, pour acrylamide mix on the bottom half of the tall plate and slowly lower the short plate, making sure that air bubbles are eliminated. Elevate the top of the gel so that it is approximately 5° from horizontal. Clamp the sides with large binder clips and insert the comb. Keep the gel in this position until polymerized.

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# *Escherichia coli* and *Salmonella*

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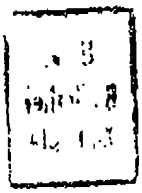

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# DNA Repair Mechanisms

W. DEAN RUPP

# 121

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## INTRODUCTION

We are now very used to seeing the familiar Watson-Crick structure for normal DNA in which the two strands interact through hydrogen bonds connecting the complementary bases. This normal structure can be altered or damaged in a variety of ways, and all cells have a collection of methods and strategies to overcome these imperfections. In this chapter, we consider the types of alterations observed in DNA and how *Escherichia coli* responds to the presence of these alterations to overcome the potentially deleterious consequences of DNA damage.

## TYPES OF DAMAGE

A variety of defects can be present in DNA. These defects can be introduced by spontaneous in situ reactions, insults from external physical and chemical agents, and reaction with intermediates present during normal metabolism (17, 45, 74). In this chapter, we consider some representative examples of different types of damage, but a comprehensive list of all known varieties of damage is beyond the scope of this review (see reference 17 for more details).

### Mismatches

Mismatches involve an incorrect base in one strand that does not pair correctly with a base in the other strand. Replication errors can result in the incorporation of an incorrect base into the new daughter strand. The replicating fork contains many accessory proteins in addition to the main polymerase that result in a great increase in the fidelity of replication. These other factors that increase fidelity of replication are discussed in more detail in chapters 50, 89, and 118 of this volume. However, a few replication errors are still made and are left behind after replication.

Spontaneous deamination of bases in DNA occurs at a low but significant rate. Two of the most frequent events are the deamination of cytosine to form uracil (which then codes like thymine) and the deamination of adenine to form hypoxanthine (which codes like guanine) (17, 45).

### Missing Bases (AP Sites)

Missing bases in DNA, also called abasic or AP sites (AP is an abbreviation for both apurinic and apyrimidinic), are the result

of cleavage of the bond linking the base to the deoxyribose in the sugar-phosphate backbone of DNA. Such cleavage can occur spontaneously, most often in the case of purines, or after alkylation of the base which causes the bond linking the base to the sugar-phosphate backbone to become more labile (17, 45). AP sites can also be formed in DNA as intermediates in the repair process when a DNA glycosylase removes a particular base from the backbone. This process is considered in more detail later in this chapter.

### Altered Bases

Altered bases in DNA can be formed after irradiation with UV or ionizing radiation and also as a consequence of exposure to certain chemicals. The effects of radiation can be direct, that is, a consequence of the interaction of the radiation directly with DNA, such as the formation of cyclobutane pyrimidine dimers after exposure of the DNA to UV light. Ionizing radiation can cause alterations either directly by the direct deposition of energy in the DNA or indirectly by the action of reactive solvent species such as radiation-produced radicals and ions.

DNA can also become damaged after reaction with a variety of chemicals. Some chemicals interact directly to damage DNA, while others may need to be converted to a more reactive species. In some cases, the cell is responsible for converting a chemical to an active form, while in other cases (such as psoralen and its relatives), the chemical is activated by light only after it has bound noncovalently to DNA (17).

Other possible chemical sources for DNA damage come from the metabolic intermediates of the cell itself. For example, *S*-adenosylmethionine, the metabolic methyl donor, is able to transfer methyl groups to DNA (74). Intermediates in oxidative processes are capable of oxidizing DNA bases to make them noninstructive or mutagenic, while some enzymes, such as DNA methylases, can cause deamination of cytosine to uracil under certain conditions (102, 131).

### Single-Strand Breaks

Single-strand breaks (and sugar damage that leads to breaks) can be introduced by several means, including ionizing radiation, various chemicals, and attack by nucleases.

### Double-Strand Breaks

Double-strand breaks can result from the chance occurrence of two single-strand breaks that occur close to each other in the complementary strands or can result from a single event, as in deposition by ionizing radiation of a large amount of energy that is sufficient to break both strands.

### Cross-Links

Cross-linking of complementary strands occurs with some chemicals, particularly with bifunctional alkylating agents but also with some chemicals such as psoralen derivatives, which intercalate into the DNA backbone and have two reactive sites that react with pyrimidines in the complementary strands with high efficiency when the chemical is photoactivated. With the psoralens, the activating wavelength is longer than that absorbed by the DNA itself, so psoralen can be added to form monoadducts and cross-links with minimal other photodamage to the DNA when appropriate care is taken with the experimental protocol (17).

## POSSIBLE STRATEGIES FOR DNA REPAIR

In principle, there are several strategies that cells could use to repair the effects of damage to DNA, and in fact, during evolution, *Escherichia coli* has accumulated a battery of responses that includes a variety of these possibilities. The simplest and most direct mechanism is reversal of the effects of the damage directly without otherwise altering the DNA structure. Two protein reactions that use this strategy are the methyltransferase activity on $O^6$-methylguanine ($O^6$-MeG), which removes the methyl to regenerate G in the DNA, and the photolyase reaction, which reverses cyclobutane pyrimidine dimers to regenerate the two original pyrimidines. Other repair or recovery processes use several different specific mechanisms to remove damaged segments from one strand and then utilize the redundancy of information in the two strands of DNA to reconstruct the original sequence by copying the undamaged strand. In other cases, the actual damaged portion may not need to be removed from the DNA molecule; recombinational exchanges can restore information to at least one strand of the DNA even though the correct information was previously absent from both strands.

## DIRECT REVERSAL OF DAMAGE

Direct reversal of damage by repair proteins without any breakage of the sugar-phosphate backbone occurs in several cases.

### DNA Repair Methyltransferase

DNA repair activity removes methyl groups from several specific sites in DNA, including $O^6$-MeG, $O^4$-MeT, and the methyl phosphotriesters that result from methylation of the phosphate in the sugar-phosphate backbone. This activity was first identified when the phenomenon of adaptation was studied in detail (76, 90). During adaptation, exposure of cells to low concentrations of methylating agents (and other alkylating agents) causes the cells to develop resistance to the lethal and mutagenic effects of subsequent exposure to much higher amounts of the adapting agents. Biochemical studies revealed that the disappearance of $O^6$-MeG from the DNA accompanied this phenomenon. More extensive biochemical and genetic studies showed that during this process, a methyl group is actually transferred from the $O^6$-MeG in DNA to a specific 39-kDa protein, leaving behind a normal G in the DNA. Furthermore, this protein is also involved in the regulation of the adaptation response. The 39-kDa protein is the gene product of the *ada* gene and is designated Ada. The role of Ada in regulation is discussed in a later section. The Ada protein has two domains that act as acceptors in methyltransferase reactions from methylated sites in DNA (1, 56, 89). The site to which a methyl is transferred from $O^6$-MeG in DNA is a cysteine (Cys-321) near the carboxyl terminus. In addition to this active site on the Ada protein, the methyl group from methyl phosphotriesters in DNA can be transferred to a different site (Cys-69) in the Ada protein near its amino terminus, and methylation of this amino-terminal site correlates with the function of Ada as a transcriptional activator. An interesting property of the Ada protein is that when a site on Ada is methylated, the methyl group remains indefinitely. This means that the protein is used

once for each site and does not recycle in the catalytic way that is typical for enzymes. Thus, to remove many methyl groups from DNA, a separate protein must be used for each methyl that is removed. (More precisely, each polypeptide can remove one methyl from an alkylated base and one methyl from a methyl phosphotriester, since the two methyl-accepting sites are separate and distinct in their locations and functions.) Because the transfer of the methyl group from DNA to Ada is a one-way process in which the methyl remains permanently attached to Ada, Ada has been called a suicide DNA repair protein.

Recent studies have demonstrated the presence of a second DNA repair methyltransferase that removes methyl groups from $O^6$-MeG of the DNA of cells in which the *ada* gene has been inactivated by mutation. This protein is now known to be the gene product of the *ogt* gene (shorthand for DNA $O^6$-MeG transferase), which has been identified and mapped at min 30.1 on the *E. coli* chromosome (69, 114). In contrast to the regulation of *ada*, the expression of *ogt* is constitutive, with no apparent activators or repressors known to be involved in its transcription.

### Cyclobutane Dimer Photolyase (Photoreactivating Enzyme)

Another reaction in *E. coli* that operates by the direct reversal of damage in the DNA is that of the DNA photolyase (23, 31, 32, 77, 111). The substrates for DNA photolyase are *cis-syn* cyclobutane pyrimidine dimers (Pyr<>Pyr) that are formed in UV-irradiated DNA in which two pyrimidines are adjacent in the same strand. A large number of careful, detailed studies have shown quite clearly how this enzyme works. The *E. coli* DNA photolyase is a monomeric polypeptide of 471 amino acids (54 kDa). Two essential noncovalent cofactors that are also chromophores (flavin adenine dinucleotide and methenyltetrahydrofolate) play key roles in its action. Photolyase attaches to the DNA substrate in the dark. Upon exposure to light of the appropriate activating wavelength (350 to 500 nm), methylenetetrahydrofolate absorbs energy from the light and transfers energy to the reduced flavin adenine dinucleotide by dipole-dipole interaction. This activated intermediate then transfers an electron to the Pyr<>Pyr to split it. Further electron transfers then restore the original pyrimidines and regenerate the original form of the DNA photolyase enzyme. This polypeptide, in contrast to the DNA methyltransferase, recycles many times and operates in the typical catalytic mode that is familiar for most enzymes.

## BASE EXCISION REPAIR

Base excision repair is a repair process that begins when a damaged base is removed through the action of a DNA *N*-glycosylase that cuts the bond linking the base to the sugar-phosphate backbone of DNA (Fig. 1). This abasic (AP) site is then further processed by an AP endonuclease to cut the sugar-phosphate backbone. Some DNA *N*-glycosylases (uracil DNA *N*-glycosylase) have only glycosylase activity, while others, such as endonuclease III and MutM (formamidopyrimidine [Fapy]) glycosylase, have an associated AP endonuclease activity that is an integral part of the same polypeptide chain. After nuclease activity has cleaned up the ends, the small gap that remains is filled by a DNA polymerase; ligation of the ends then completes the base excision repair cycle. The specificity in this repair pathway is determined

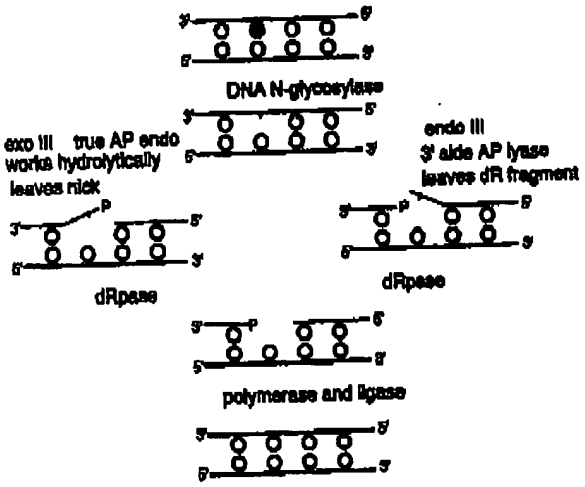


FIGURE 1 Base excision repair. A DNA *N*-glycosylase starts the process by removing a base. Some glycosylases have associated AP endonuclease (lyase) activities, while others do not and require the action of a separate true AP endonuclease such as exonuclease III or endonuclease IV. dR, deoxyribose; dRpase, deoxyribophosphodiesterase.

by the properties of the particular DNA *N*-glycosylase that initiates the process (Table 1). A battery of different glycosylases provides the cell with the ability to recognize and repair many of the most common alterations in DNA. Some of these DNA *N*-glycosylases (particularly uracil DNA *N*-glycosylase) are highly specific for a particular substrate, while others (Fapy glycosylase and endonuclease III) have a wider range of substrates that includes several different damaged bases. Several years ago, researchers anticipated finding many different glycosylases with high specificities for their substrates, but it is now recognized that some of the enzymes act on multiple substrates, so these various activities are actually performed by a relatively small number of separate DNA *N*-glycosylases.

### Glycosylases

**Uracil DNA *N*-Glycosylase.** Uracil is produced in DNA by deamination of cytosine and is removed by uracil DNA *N*-glycosylase, the product of the *ung* gene. This enzyme is very specific for U as a substrate. As mentioned above, this enzyme is a simple glycosylase and does not have associated AP endonuclease or lyase activity.

**Tag (3-MeA DNA Glycosylase I) and AlkA (3-MeA DNA Glycosylase II).** Alkylation products in DNA are substrates for at least two different 3-MeA DNA glycosylases that are products of the *tag* and *alkA* genes (7). Tag has greater selectivity than AlkA, removing 3-MeA much better than 3-MeG, while AlkA has a broader specificity and removes them both quite efficiently. AlkA is also known to remove hypoxanthine from DNA (85). (Hypoxanthine is a mutagenic product formed by deamination of A.) The expression of the *tag* gene is constitutive, while the *alkA* gene product is under the control of Ada. This coordinated expression of *alkA* and *ada* is noteworthy in that the two gene products

TABLE 1 Genes and proteins involved in DNA repair

| Function and gene | Map position (min) | Gene product | Substrate and/or function[a] |
|---|---|---|---|
| Direct reversal of damage | | | |
| *ada* | 49.7 | Ada | Alkyltransferase ($O^6$-G, $O^4$-T, and alkylphosphotriesters) transcriptional activator of genes with Ada box |
| *ogt* | 30.1 | $O^6$-G methyltransferase | $O^6$-G alkyltransferase |
| *phr* (*phrB*) | 16.0 | DNA photolyase | Splits cyclobutane pyrimidine dimers (in light) |
| Glycosylases | | | |
| *ung* | 58.3 | Uracil DNA glycosylase | Uracil (deamination product of C) ss > ds > loops |
| *tag* | 80.0 | Tag, 3-MeA DNA glycosylase I | 3-MeA>>3-MeG |
| *alkA* | 46.2 | AlkA, 3-MeA DNA glycosylase II | Various alkylpurines, including 3-MeA and 3-MeG, and hypoxanthine (controlled by Ada) |
| Glycosylases with associated AP endonuclease (lyase) | | | |
| *mutM* (*fpg*) | 82.1 | MutM, Fapy glycosylase, GO glycosylase, FPG, AP lyase | Glycosylase with associated AP endonuclease activity; acts on Fapy, GO, and other oxidized purines; also acts on some pyrimidine products |
| *nth* | 36.8 | Endonuclease III | Glycosylase with associated AP lyase; acts primarily on pyrimidine hydrates |
| | | Endonuclease VIII | Glycosylase and AP lyase similar to endonuclease III; thymine glycol, dihydrothymine, β-ureidoisobutyric acid, urea, AP sites (but not reduced AP sites) (probably nicks on 3′ side of lesion) |
| *mutY* (*micA*) | 66.8 | MutY glycosylase (adenine mispair glycosylase) | Removes A from A:8-oxoG mispairs that are replication products of 8-oxoG (also removes A from A:G, A:C, and A:oxoA mispairs) |
| AP endonucleases | | | |
| *xthA* | 39.4 | Exonuclease III | AP endonuclease; hydrolyzes bond 5′ to damaged site, leaving deoxyribose attached to 5′ terminus; also acts on site with urea residue |
| *nfo* | 48.4 | Endonuclease IV | AP endonuclease hydrolyzes bond 5′ to lesion, leaving lesion on 5′ terminus; also works on α-deoxyadenosine (radiolysis product) and THF derivatives (abasic site model) |
| Mismatch repair | | | |
| *vsr* | 43.8 | Vsr endonuclease | Cuts on 5′ side of T in TG mismatches at Dcm sites when 5-MeC is deaminated to form T |
| *mutH* | 63.9 | MutH | Endonuclease component of MutHLS methyl-directed mismatch repair system that cuts at hemimethylated sites |
| *mutL* | 94.7 | MutL | Interacts with MutH and MutS to coordinate their action |
| *mutS* | 61.5 | MutS | Mismatch recognition component of MutHLS methyl-directed mismatch repair system |
| *sbcB* (*xonA*) | 44.9 | Exonuclease I | Involved in MutHLS mismatch repair |
| *recJ* | 65.4 | RecJ | Involved in MutHLS mismatch repair (and recombination) |
| Cleansing of precursor pool | | | |
| *mutT* | 2.4 | MutT | Hydrolyzes 8-oxodGTP to 8-oxodGMP before it can be misincorporated into DNA |
| Nucleotide excision repair | | | |
| *uvrA* | 92.0 | UvrA | UvrABC acts on wide variety of bulky lesions, DNA cross-links, etc., to make dual incision on each side of lesion |
| *uvrB* | 17.6 | UvrB | UvrABC acts on wide variety of bulky lesions, DNA cross-links, etc., to make dual incision on each side of lesion |
| *uvrC* | 43.0 | UvrC | UvrABC acts on wide variety of bulky lesions, DNA cross-links, etc., to make dual incision on each side of lesion |
| *mfd* | 25.3 | Mfd | Transcription coupling factor in UvrABC strand-specific repair of transcribed strand |
| *uvrD* | 86.1 | UvrD, helicase II | Participates in MutHLS mismatch repair and also UvrABC nucleotide excision repair |
| Postreplication repair, SOS regulation and translesion synthesis | | | |
| *recA*[b] | 60.8 | RecA | Strand exchange between homologous DNAs |
| *lexA* | 91.6 | LexA | Repressor of SOS Rec-Lex genes |
| *umuC* | 26.5 | UmuC | Involved in SOS mutagenesis and translesion synthesis |
| *umuD* | 26.5 | UmuD | Involved in SOS mutagenesis and translesion synthesis |
| Redox control of transcription | | | |
| *soxR* | 92.1 | SoxR | Redox sensor |
| *soxS* | 92.1 | SoxS | Transcriptional activator (with redox-activated SoxR) |
| General repair | | | |
| *polA* | 87.1 | DNA polymerase I | Involved in many repair processes |
| *lig* | 54.5 | DNA ligase | Joins strands to finish many repair and recombination processes |

[a] ss, single stranded; ds, double stranded; THF, tetrahydrofuran.
[b] For other *rec* genes involved in repair and recombination, see chapter 119 in this volume.

repair different types of alkylation damage by different mechanisms. The linked regulation of these two proteins thus optimizes the repair of several diverse lesions that are likely to be formed in DNA by a single alkylating agent.

### Glycosylases with Associated AP Endonuclease (Lyase) Activities

**MutM (Fapy) Glycosylase.** Oxidative damage to purines and pyrimidines is a consequence of exposure to metabolic intermediates and certain other chemicals as well as exposure to radicals produced by ionizing radiation. Two proteins, Fapy glycosylase and endonuclease III, are glycosylases with associated AP endonuclease activities that remove many of the most significant oxidative lesions. Although there is some overlap in the substrates of the two proteins, endonuclease III acts primarily on pyrimidine products, while Fapy glycosylase acts primarily on damaged purines. Fapy is formed when the imidazole ring of a damaged purine is opened. Fapy DNA glycosylase is known by various names, including 8-oxoguanine (8-oxoG) DNA glycosylase, Fapy glycosylase, FPG protein, and MutM protein. It has *N*-glycosylase activity that releases 8-oxoG and Fapy from oxidatively damaged DNA (8, 20, 117). (Fapy is a purine oxidation product in which the five-member ring has been opened.) This enzyme also releases 5-hydroxy cytosine and 5-hydroxy uracil, common pyrimidine oxidation products that are now known to be removed by endonuclease III as well as by Fapy glycosylase (22).

**Endonuclease III.** Endonuclease III was originally identified as an enzyme that cut DNA irradiated with high fluences of UV light (66). It is now known that endonuclease III acts as a DNA glycosylase on pyrimidine derivatives with rings that are saturated, contracted, and rearranged. These derivatives include thymine glycol and other 5,6-dihydropyrimidines (13). 5-Hydroxy cytosine and 5-hydroxy uracil are other common pyrimidine oxidation products that are now known to be removed by both endonuclease III and Fapy glycosylase (22).

**Endonuclease VIII.** Endonuclease VIII, a recently described activity, is similar to endonuclease III in its substrate range and in the fact that it is likely to have an associated AP lyase activity that nicks on the 3′ side of the lesion, because it generates blocked 3′ ends (50).

### AP Endonucleases

**Exonuclease III and Endonuclease IV.** AP endonucleases are divided into two categories: the true AP endonucleases such as exonuclease III (class II, 5′ nucleotidyl hydrolases) hydrolyze the 5′ phosphodiester bond adjacent to an AP site, generating a nick with typical 3′ hydroxyl and 5′ phosphoryl termini, while the AP lyases (class I), of which endonuclease III and MutM (Fapy glycosylase) are examples (8, 37), catalyze the cleavage of the phosphodiester bond 3′ to an AP site by a mechanism that cleaves the deoxyribose moiety and generates termini with normal 5′ phosphoryls but leaves on the 3′ end only an altered deoxyribose that is a block to chain elongation by DNA polymerase I. Exonuclease III also recognizes and cuts at urea residues in oxidized DNA (36). Inside the cell, the two major true AP endonuclease activities are exonuclease III and endonuclease IV. This conclusion is based on studies of UV-irradiated T4 bacteriophage in which the repair is initiated by the *denV* gene of T4, which has a phage-encoded DNA glycosylase that is specific for pyrimidine dimers. The UV-irradiated phages showed reduced survival on the *xth nfo* double mutant but normal survival when plated on the single mutants, indicating that either protein provided enough activity for optimal survival (86).

The deoxyribose fragment left at the end of a strand by the action of an AP endonuclease or AP lyase needs processing, because it is a block for gap filling by a DNA polymerase. An activity that removes this abasic residue was described by Franklin and Lindahl (16) and named DNA deoxyribophosphodiesterase. In subsequent studies, the two groups attempting to assign this activity to a specific enzyme came to different conclusions. Sandigursky and Franklin (81–84) assigned this activity to exonuclease I, while Dianov et al. (14) reported that the deoxyribophosphodiesterase is associated with the RecJ protein.

**SoxRS Control of Endonuclease IV.** A number of functions that are responsive to oxidative stress are under SoxRS control (see chapter 95 in this volume). Of these, *nfo*, the structural gene for endonuclease IV, is the only one that is directly concerned with DNA repair. The regulatory control is complex in that SoxR is produced constitutively and senses the redox state of the cell by a mechanism that is only vaguely understood. Under conditions of oxidative stress, SoxR becomes a transcriptional activator of *soxS*, whose gene product in turn activates the *nfo* gene to increase the synthesis of endonuclease IV (41, 59, 130).

## GO (OXIDIZED GUANINE) REPAIR

Oxidized guanines must be a significant problem for the cell, as evidenced by the fact that *E. coli* has devised a multifaceted approach for dealing with the products of guanine oxidation (Fig. 2). This approach has been called the GO system and includes two different glycosylases (MutY adenine glycosylase and MutM Fapy glycosylase) along with MutT, a novel 8-oxoguaninedeoxyribotriphosphatase (8-oxodGTPase) that acts to cleanse the precursor pool. Oxidized guanine precursors of DNA can be misincorporated into DNA opposite A during DNA replication. To decrease this probability, the cells have developed a particular enzyme, the MutT protein, that converts 8-oxodGTP to the monophosphate form before it becomes incorporated into DNA (46). When a G in DNA is oxidized to 8-oxoG, this altered base is a substrate for the glycosylase that is the product of the *mutM* gene. This protein is known by various names, including Fapy glycosylase, Fapy DNA glycosylase, 8-oxoG DNA glycosylase, FPG protein, and MutM protein (8, 20, 116, 117). It has *N*-glycosylase activity that releases 8-oxoG and Fapy from oxidatively damaged DNA. This protein is one of those glycosylases that also has an associated AP endonuclease activity that cuts the phosphodiester backbone. Exonuclease processing of the gap generated by MutM creates a substrate that can then be restored with DNA polymerase and ligase. Replication of a template with 8-oxoG can result in the significant misincorporation of A opposite the 8-oxoG. In yet another strategy for coping with the consequences of guanine oxidation, *E. coli* has an additional enzyme, the MutY protein, which is an adenine DNA glycosylase with associated AP lyase that acts to remove the A from DNA structures containing mismatches of A with 8-oxoG (53, 57, 119). Repair of this structure results in the formation of a structure with C paired with 8-oxoG, which is the substrate for the MutM glycosylase (52). This system is fascinating because it demon-

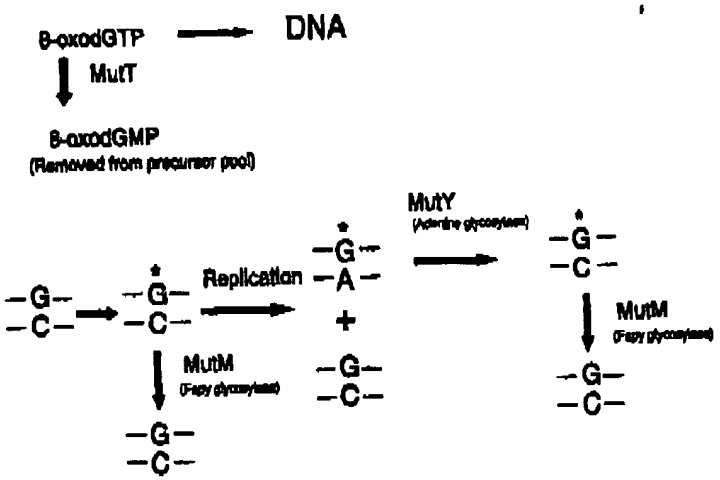


FIGURE 2 GO system for repair of oxidized G. Several enzyme activities work in concert to overcome the effects of guanine oxidation on different stages, including the precursor pool and double-stranded DNA, before and after replication (see text for details).

strates the response of the cell to damage in the DNA precursors, in the DNA itself, and in the aberrant products resulting from the replication of DNA with the oxidized guanine lesion.

## STRAND BREAK REPAIR

Many of the single-strand breaks introduced by X irradiation are repaired very rapidly with a half-life of a few seconds, as in the joining of newly synthesized Okazaki fragments into longer DNA molecules (27, 28). Thus, when there is no intrinsic block to joining, repair of single-strand breaks is very rapid and is carried out by DNA polymerase I and DNA ligase. Most breaks made by X rays are more complex and may even be formed indirectly as a consequence of chemical or enzymatic conversion of other radiation products. Several of the most common single-strand breaks inferred to be present after irradiation have been studied in a ϕX174 replicative-form transfecting DNA model system (35). Ligatable nicks with 3′ hydroxyl and 5′ phosphate termini were not lethal. Other breaks were made from substrates with thymine glycols, urea residues, or abasic (AP) sites by treatment with endonuclease III to generate a single-strand nick with a 3′ α,β-unsaturated aldehyde (4-hydroxy-2-pentenal). Single-strand breaks with a 5′ deoxyribose or a 5′ deoxyribosylurea were made by treating the AP or urea substrates with endonuclease IV. These enzyme-generated breaks had inactivation efficiencies of 0.12 to 0.14, which is similar to the inactivation of the damaged molecules without the enzyme treatment.

## MISMATCH REPAIR

Mismatch repair is the mechanism that processes mismatches in which two undamaged normal bases that cannot form the classic Watson-Crick base pairs are present in the complementary strands. The most frequent origin of such mispairs is the incorrect insertion of nucleotides by polymerases during replication. In the case of mispairs, the cell faces the dilemma of having an undamaged normal base in each strand and requires a mechanism to distinguish the "right" one from the "wrong" one. In order to make this discrimination, the cell uses differential methylation of the two strands. The A in GATC sites is methylated by the Dam methylase subsequent to replication at a rate that ensures that most or all sites are methylated by the time the next replication occurs but is slow enough so that a significant number of sites are only hemimethylated shortly after replication (i.e., only the old template strand is methylated) (19, 54; also see chapter 53 of this volume).

The cells simultaneously detect the presence of a mismatch and a hemimethylated site through a complex dual sensing mechanism that requires the products of four genes: *mutH*, *mutL*, *mutS*, and *uvrD* (alternately named *mutU*, whose gene product UvrD is also known to be helicase II). The first step is the detection of a mismatch through the binding of MutS to the mismatch. MutL then binds and in some way activates MutH endonuclease activity, which interacts with hemimethylated sites to cut the unmethylated strand at the GATC site. Unidirectional exonucleolytic degradation from the cut toward and past the mismatch results in preferential removal of the mismatched nucleotide from the undermethylated (i.e., the newly replicated) strand. The specific enzymes participating in this exonucleolytic degradation depend on the orientation of the cleaved GATC site relative to the mismatch. When the cut at the GATC site is on the 5′ side of the mismatch, exonuclease VII or RecJ provides the exonuclease function; in the other case, when the cut at the GATC site is 3′ to the mismatch, exonuclease I does the digestion. In either case, the degraded strand (which may be as long as several kilobases) is replaced by using DNA polymerase III holoenzyme and single-strand binding protein, with repair being completed by ligation of the resulting nick by DNA ligase. This mechanism ensures that the information retained at the site of a mismatch will be that of the old original strand and that the incorrect nucleotide inserted at replication will be the one removed, thus maintaining the correct DNA sequence (Fig. 3).

MutS and MutL proteins also reduce recombination between related DNAs that are not exactly homologous, and they account for much of the barrier to genetic exchange between related organisms such as *E. coli* and *Salmonella* spp. This phenomenon has been studied in a cell-free system with purified components, including MutS, MutL, and RecA, where it was demonstrated that

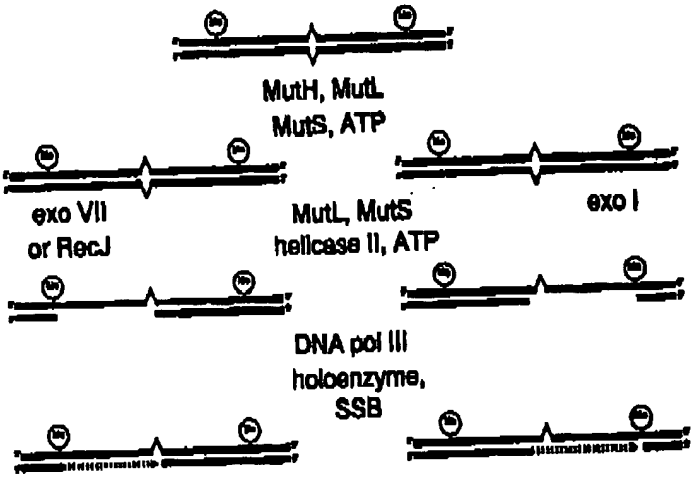


FIGURE 3 Mechanism of methyl-directed mismatch repair (adapted from references 19 and 54).

MutS and MutL greatly reduce the strand exchange between molecules with several percent mismatches but do not inhibit the exchange of completely homologous DNAs. Presumably, the binding of MutS and MutL to mismatches effectively blocks the orderly exchange of strands by RecA (68, 129).

## VSP REPAIR

VSP (very short patch) repair (Fig. 4) operates on the specific sequences -CC(A/T)GG-, in which the second cytosine is methylated at position 5 by Dcm (42). When 5-MeCs are deaminated, they are converted to thymine. Consequently, they are not recognized by uracil glycosylase, the enzyme that recognizes and removes uracils that are formed during deamination of normal unmethylated cytosine. The VSP system specifically recognizes thymine-guanine mismatches in the sequence context that is the substrate for methylation of C by the Dcm methylase and then repairs the thymine-containing strand by a combination of Vsr endonuclease (the *vsr* gene product), repair synthesis, and ligation that results in a replacement patch of only several nucleotides, accounting for the term "very short patch repair." The Vsr nuclease provides the specificity in this reaction, causing incision only on the 5′ side of the T in the thymine strand of the thymine-guanine mismatch and thus avoiding mutations caused at this site from the deamination of 5-MeC (24, 42; see also chapter 53 in this volume).

## NUCLEOTIDE EXCISION REPAIR

### UvrABC

The nucleotide excision repair process initiated by the UvrABC nuclease operates on a variety of diverse DNA lesions, particularly those that are large and bulky and cause significant distortions in DNA. The advantage to the cell of this system is that while other systems depend on specific recognition of a particular lesion or type of damage, the UvrABC excinuclease removes many different damages with widely different chemical and structural properties, presumably by sensing subtle changes in the DNA structure rather than by making specific protein-lesion contacts. Consequently, the single UvrABC system works on many types of damage, even those to which the cell may never have had any previous exposure (9, 64, 79, 99).

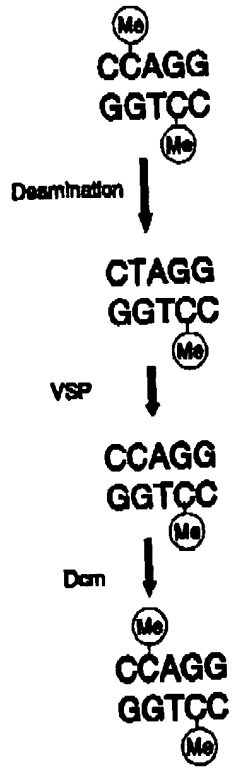


FIGURE 4 VSP repair of mismatches resulting from the conversion of 5-MeC to T by deamination. This is a specialized repair process that occurs in sequences where cytosine is methylated because of its presence in the Dcm recognition sequence. (Deamination of cytosines in other sequences converts them to U, the substrate for uracil glycosylase.) The specificity is provided by the Vsr nuclease, which makes a strand-specific cut immediately 5′ to the T in the TG mismatch (24, 42; see text for details; also see chapter 53 in this volume).

The UvrABC complex makes a double incision on the strand with the lesion. One incision is 3 to 4 nucleotides (nt) on the 3′ side, and the other is 7 nt on the 5′ side, a site far enough removed from the actual lesion that the incisions are located in the DNA duplex at places that are little altered by the lesion's presence (79, 132). While these flanking incisions are normally tightly coupled together, further analysis has demonstrated that the UvrABC reaction is a complex cascade of sequential reactions. The overall reaction requires ATP, and much is now known about individual steps that require ATP binding and/or hydrolysis, although interpretation is complicated by the fact that UvrA has multiple nucleotide-binding motifs and UvrB has only one (17, 21, 78, 80).

UvrA dimerizes to form a $UvrA_2$ dimer, which then interacts with UvrB to form a $UvrA_2B$ complex. UvrA by itself has affinity for DNA, but UvrB does not bind to DNA unless UvrA is present. UvrA has the ability to interact specifically, and footprints at damaged sites are observed with UvrA alone (6, 58, 120). However, tight, stable complexes with damaged DNA are formed only when both UvrA and UvrB are present. Interaction with UvrC then occurs, and the dual incision takes place. Studies by Shi et al. (104) indicate that in the stable complex formed in the presence of UvrA and UvrB, the DNA is sharply bent, and UvrA is probably no longer present. In this complex, UvrB must be in very close proximity to the damaged site, because when a psoralen monoadduct is used as the substrate, subsequent exposure to near-UV light causes the psoralen monoadduct to be photo-cross-linked to the UvrB protein. The dual incisions require both UvrB and UvrC and are normally tightly coupled. Through the use of site-specific mutants in *uvrB* and *uvrC*, it has now been concluded that the first incision occurs on the 3′ side of the lesion and uses a catalytic site in UvrB, while the second incision, on the 5′ side, utilizes a catalytic site in UvrC (43, 44).

The role of ATP binding and hydrolysis is complex, because UvrA has multiple nucleotide-binding domains, while UvrB has a cryptic ATPase activity that is associated with a limited helicase activity of the $UvrA_2B$ complex (21, 62). Seeley and Grossman (94, 95) observed that site-specific mutagenesis of the nucleotide-binding site in *uvrB* simultaneously inactivates the helicase activity and prevents the formation of tight specific preinitiation complexes with damaged DNA but does not affect formation of the $UvrA_2B$ complex and its binding to undamaged DNA. Although it has been suggested that this helicase activity may be involved in translocation of the $UvrA_2B$ complex along undamaged DNA searching for lesions (21, 62), other studies (I. Gordienko and W. D. Rupp, unpublished data) show that the helicase activity is very limited in the length of oligonucleotide that can be released. This activity may thus be a very localized action in which the complex is flexing and probing the DNA to determine whether there is a lesion present and then, if there is, to correctly assemble the components in preparation for incision. The localized bending and unwinding of DNA associated with locating the lesion and precisely loading UvrB may account for the observed separation of positive and negative domains when UvrA and UvrB act on supercoiled DNA (33).

### How Does UvrABC Recognize a Substrate?

Although it is clear that many repair enzymes such as the alkyltransferases, DNA photolyase, and the DNA glycosylases "recognize" their substrates by interacting with them in highly specific protein-lesion contacts, the variety of lesions recognized by the UvrABC system requires a much different process. Although the UvrABC system is the only system for removing a variety of bulky DNA adducts, other types of damage that are handled by some of the other more specific systems can also be removed by UvrABC, and it has been suggested that UvrABC actually "repairs everything" (78). Since a static interaction of protein with substrate seems inadequate to account for the wide variety of substrates utilized by UvrABC, it was suggested that recognition of lesions might require a more active process in which UvrAB flexes and distorts DNA while looking for an atypical response, much as a physician flexes an injured limb while checking on its range of motion (65). The results showing the extreme bending of DNA in the UvrB complex are consistent with this expectation (104). Snowden and Van Houten observed (106, 120) that the formation of the stable UvrAB complex at a damaged site is the limiting step for incision. However, with a psoralen DNA cross-link (58) and with cisplatin DNA adducts (124), other steps later in the reaction sequence can be rate limiting for incision. A model for the overall reaction cascade is presented in Fig. 5. The overall specificity and selection for substrates are determined by the net contribution of all the various individual steps, not by one single highly selective reaction. This can account for the high selectivity of the overall process, which is greater than the selectivity at any one specific step, presumably because different parameters of the structure can be tested at different stages. For example, the parameter being tested at the first step might be the presence of a "weak" place in the helix that allows the helix to be bent or deformed at a particular site. Subsequent steps involving the UvrAB probing of a limited region might then provide a higher-resolution test to determine whether there actually is at that position a lesion that can be converted into a complex suitable for incision. For example, after comparing results for model substrates containing a bulky lesion (AAF) that is a particularly good substrate for incision with results for a substrate containing a mismatch that is incised very infrequently if at all, Gordienko and Rupp (unpublished data) suggested that while both substrates can initially be recognized by $UvrA_2B$, the reaction cascade with the AAF substrate proceeds well, while the reaction with the mismatch is usually aborted and only rarely proceeds to successful incision. With a psoralen monoadduct, the cascade proceeds through incision; with a psoralen cross-link, a similar stable complex is formed in the presence of UvrA and UvrB; and in the presence of UvrC, incision was inefficient in relaxed DNA but rapid when the DNA was negatively supercoiled (58). Visse et al. (122–124) also noted with cisplatin adducts that there were rate-limiting steps after the formation of the UvrB complex at the damaged site. Some model substrates may mimic intermediates in the overall process. Substrates with bubbles offer preferential sites for the attachment of UvrAB to duplex DNA (Gordienko and Rupp, unpublished data), and structures with no damage and 5′ single-strand extensions result in cutting near the 3′ end and a reaction that appears to mimic that involved in cutting on the 5′ side of lesions, which cannot normally be studied, because it occurs in a concerted reaction following the initial cut on the 3′ side of a lesion (71).

### Mfd and Preferential Repair of the Transcribed Strand by UvrABC

Mellon and Hanawalt (51) demonstrated that transcription of a gene increases the repair of the transcribed strand. Selby et al. (98) showed that this enhanced repair is due to the product of the *mfd* gene, which is involved in the phenomenon of "mutation frequency decline" (126). The cloning of *mfd* and the study of its gene product in purified systems have produced a general under-

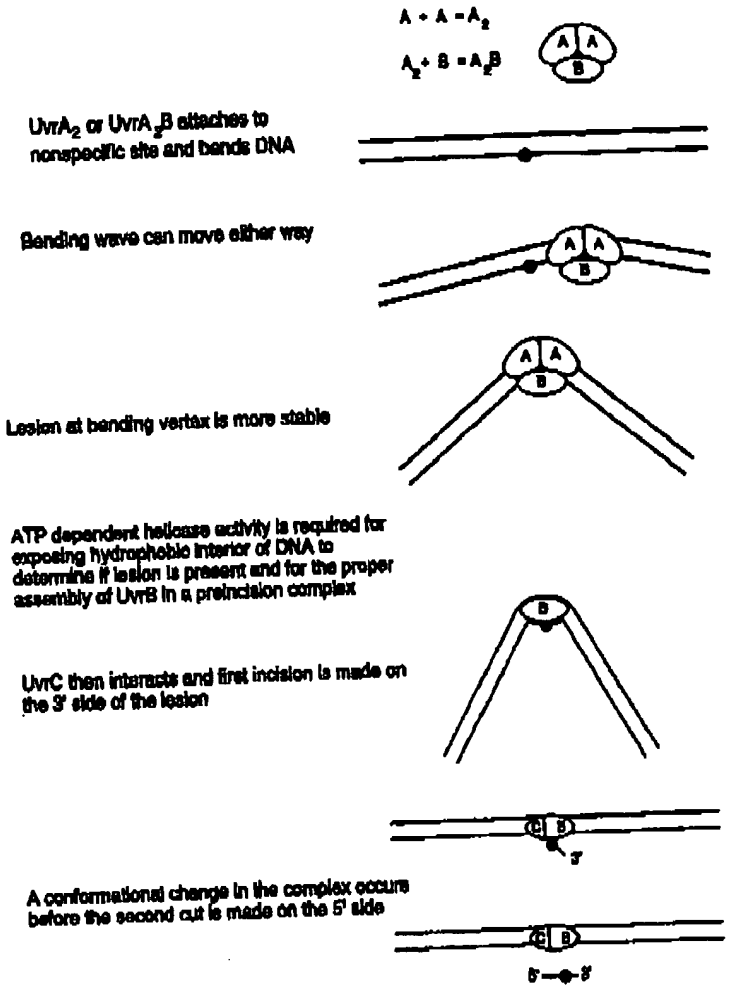


FIGURE 5 Nucleotide excision repair: UvrABC mechanism.

standing of what happens at lesions in the transcribed strand that block RNA polymerase (96, 97). Mfd protein interacts directly with RNA polymerase stalled at lesions in the transcribed strand to displace the RNA polymerase. Mfd also has affinity for UvrA and recruits UvrA to the damaged site, and UvrA in turn interacts with UvrB, loading it to form a preincision complex that after interaction with UvrC results in the removal of the blocking lesion.

## REGULATORY CONTROL OF DNA REPAIR GENES

### Inducible Error-Prone (SOS) Repair

SOS repair depends on *umuCD* genes to bypass damage and is discussed in chapters 89 and 118 in this volume. Other repair genes are also under the control of the LexA repressor, which is inactivated by self-proteolysis that is stimulated by interaction with RecA bound to single-stranded DNA generated during replication of damaged DNA (17, 87).

### Ada and the Adaptive Response

Ada, the 39-kDa product of the *ada* gene, is an important gene regulator in addition to having a direct role as a methyl acceptor during DNA repair (1, 89, 118). As pointed out before, Ada has two separate methyl-accepting domains: the N-terminal domain of the protein accepts methyls from methyl-phosphotriesters in DNA, while the C-terminal domain contains the site that receives the methyls transferred from the alkylated DNA bases $O^6$-MeG and $O^4$-MeT. The N-terminal domain of Ada is now known to be the part of the protein most important for the regulatory activity in modifying transcription (Fig. 6). When the N-terminal domain of Ada is methylated as a consequence of its methyltransferase activity acting on DNA phosphotriesters, Ada becomes a transcriptional activator for its own gene *ada* and also for the *alkA* gene, which codes for the synthesis of 3-MeA DNA glycosylase II. The methylated Ada protein binds to a regulatory sequence, designated the Ada box, that precedes the regulated genes. (Ada protein that is not methylated at Cys-69 in its N-terminal domain binds much less strongly to the Ada box.) Interaction of the bound Ada with RNA polymerase increases transcription of the gene, thus increasing the synthesis of the gene products Ada and AlkA. This mechanism explains the induction of these genes after their exposure to methylating agents. However, there is a conceptual problem in a mechanism for turning off the induction, because the Ada transmethylase activity is not catalytic but instead is a unidirectional "suicidal" process in

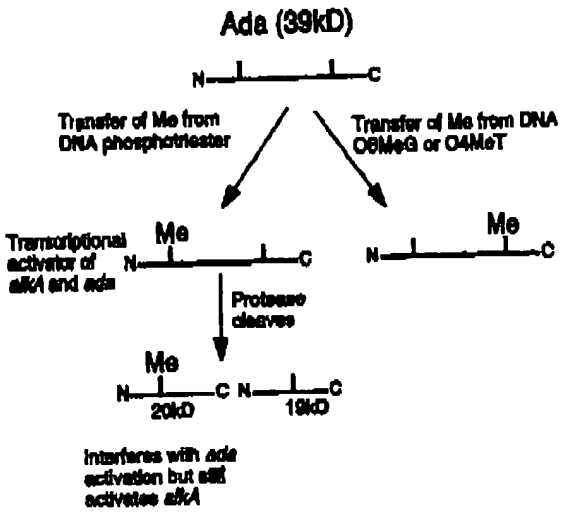


FIGURE 6 Methylation of Ada and regulation of transcription. Methyl groups from DNA are transferred to either Cys-69 or Cys-321 of the Ada protein. The OmpT protease cleaves Ada, but this reaction is not believed to be physiologically relevant, although it has been useful in studying the mechanism by which methylation of Ada converts Ada to a transcriptional activator (see text for details).

which the protein that receives a methyl group apparently retains that methyl group indefinitely and would thus be expected to continue stimulating expression even when all methyl lesions are removed from the DNA. An observation of a cellular protease activity that cleaves Ada near the middle seemed to provide a possible solution to this regulatory challenge (1). The protease cleavage generates two similarly sized fragments of Ada: a 20-kDa fragment from the N-terminal end and a 19-kDa fragment from the C terminus. The methylated N-terminal 20-kDa fragment still binds to Ada boxes, but when the fragment is bound to the regulatory region of the *ada* gene, it does not activate transcription but actually interferes with the activation observed with the intact 39-kDa Ada methylated in its N-terminal domain. Protease cleavage of the methylated Ada thus might explain how synthesis of Ada is turned off when repair of methylated DNA is complete and Ada is no longer needed. However, subsequent studies showed that the cleavage of Ada is due to OmpT, a protease found in the outer membrane (88). Since intracellular Ada is not accessible to OmpT, OmpT does not provide a physiological route for turning off the expression of those genes activated by methylated Ada.

An alternate possibility for a regulatory loop comes from recent studies by Saget and Walker (75). Their experiments confirm that the methylation of Cys-69 in the N-terminal region of Ada by methyl transfer from a DNA methyl phosphotriester is the critical step in generating the transcription activator for binding to the Ada box in the *ada* promoter. However, their results indicate that Ada that is not methylated at Cys-69 does bind weakly to the Ada box and that at concentrations that are physiologically significant, such Ada interferes with the transcriptional activation of the *ada* gene. Thus, in this scenario, large quantities of unmethylated Ada could turn off the continued synthesis of itself, even though methylated Ada remained in the cell.

The regulation of the *alkA* gene by Ada differs in some details but resembles the regulation of *ada* in that methylated Ada is a transcriptional activator (103). For example, the methylated 20-kDa N-terminal product of Ada acts as a transcriptional activator for *alkA* but not *ada*. Presumably, the exact positioning of the activator at the Ada box and its specific contacts with RNA polymerase are very sensitive to slight differences in the promoter sequences and can thus result in significant differences in its activation of the two genes. The functional consequences of these differences are not yet fully understood or appreciated.

## POSTREPLICATION (RECOMBINATIONAL) REPAIR

The properties of mutants that are recombination defective demonstrate that *E. coli* has other mechanisms in addition to nucleotide excision repair that significantly enhance the survival of cells exposed to UV irradiation. More than 1,000 cyclobutane pyrimidine dimers per bacterial chromosome are required to kill a wild-type cell, while only about 50 dimers are required to kill an excision-deficient cell. In contrast, a *uvrA recA* double mutant defective in both excision repair and recombination is killed by about one lesion per chromosome (17, 25). Early work, begun in the 1960s, was designed to find out how excision-defective strains of *E. coli* were able to survive with many lesions in their DNA. Studies of the properties of DNA synthesized in UV-irradiated excision-defective *E. coli* led to the formulation of a model for recombinational repair (26, 72, 73), which was summarized in a review by the Sancars (with the assistance of Paul Howard-Flanders) (80) as follows.

> When the polymerase encounters certain nucleotide adducts, such as pyrimidine dimers, it stops replicating and reinitiates about 1000 bp beyond the adduct, thus generating a single-stranded gap that contains a modified nucleotide. This discontinuity or postreplication gap is filled in by the RecA protein, which transfers the complementary strand from the sister duplex into the gap. This model, which was formulated for *E. coli* two decades ago remains essentially unchanged.

In this section, the focus is on events that occur at a replicating fork when a blocking lesion is present in the template DNA, because those structures initially generated during replication play a key role in repair, mutagenesis, and SOS induction. Many recent studies have led to impressive advances in our understanding of details of the enzymology of DNA replication that could not even have been imagined when the early whole-cell studies were done (34, 70). Of particular mechanistic interest is the case in which the lesion is on the strand being used as the template for synthesis of the leading strand. Information that may be relevant to this situation has been compiled from a variety of sources, including studies with whole cells and with purified enzyme systems.

Much work has been invested in understanding recombinational mechanisms, but they are not considered here except to point out chapter 119 in this volume. The emphasis here is on results that are relevant to the structures that are likely to be generated at the replication fork of *E. coli* when polymerase-blocking lesions remain in the template strands.

### Replication Generates Daughter Strand Gaps

From sedimentation of newly synthesized DNA through alkaline sucrose gradients, it was estimated that the lengths of the new strands were similar to the distances between pyrimidine dimers

in the template strands (72). From the amount of radioactivity incorporated, it was calculated that the number of pieces synthesized was much larger than the number of active growing forks. Using the straightforward assumption that these pieces were generated at preexisting active forks, it followed that replicating forks had proceeded past many lesions in the template strands. The accuracy of the molecular weight estimates obtained from the distribution of radioactivity in alkaline sucrose gradients can legitimately be questioned. (In fact, after the relation between counts, sedimentation velocity, and number- or weight-average molecular weights had been derived, it was a surprise to find that the experimentally determined sizes of the newly replicated DNA agreed as well as they did with the number of lesions in the template strands and with the theoretically calculated curve. This agreement is probably a consequence of the labeling time of 1 to 10 min. Whereas Okazaki fragments are joined with a half-life of a few seconds, these fragments synthesized in UV-irradiated cells are joined with a half-life of about 15 to 20 min.) Although there may be some error in the experimentally determined sizes of these fragments, the error is almost certainly substantially less than a factor of 2. Even more significant, there was no indication of the bimodal distribution that would result from the presence of two discrete molecular weight populations, which would be expected if one strand were synthesized continuously and the other strand were synthesized in small pieces. While this result argues strongly against the continuous synthesis of either strand, it does not rule out the possibility that direct bypass synthesis might occur at a low frequency (<10%). (Other investigators have sometimes observed various extents of a small, second, faster-sedimenting peak or shoulder in the newly synthesized DNA, but this peak is most likely due to early repair rather than to continuous bypass. This faster-sedimenting shoulder is not seen in *recA* mutant cells [105]. The experiments of Ganesan [18] clearly demonstrated that the fast-sedimenting shoulder of newly synthesized DNA contains material formed by recombinational exchanges with the parental strand and is therefore not likely to be due to de novo synthesis of a continuous new strand.)

### Inhibition of DNA Synthesis by UV Irradiation: Inhibition Is Not Equivalent to a Stalled Replication Fork

Although the usual plots, particularly in wild-type repair-proficient cells, can give the impression that DNA synthesis comes to a rapid and complete stop after UV irradiation, closer analysis shows that this is not the case. Inhibition of DNA synthesis is a continuous function of UV exposure (72, 113). Under conditions in which incorporation continues at low levels for minutes or even hours, it can readily be calculated that every replication fork advancing through duplex DNA at the normal rate of 1,000 bp/s will contact a template strand lesion within a few seconds. These results are not explained satisfactorily by models in which a replication fork is proceeding at a normal rate until it reaches a lesion (either the first lesion or a particular subset of lesions) and then comes to a complete stop, but are more consistent with a model in which replication forks are slowed but not stopped. Although the analysis is complicated by DNA breakdown (25), DNA synthesis in *uvrA recA* cells also continues past many lesions. (To appreciate the extent of the breakdown problem, it can be seen from Fig. 5 of Smith and Meun [105] and Fig. 2 of Sedgwick [91] that more than 75% of the label incorporated during the first 10 min after irradiation of *uvrA recA* cells is lost during subsequent incubation for 50 to 70 min.) It is thus clear that in *uvrA recA* cells, a simple measure of the incorporation of label into DNA significantly underestimates the actual extent of synthesis, because of concurrent breakdown. My interpretation of these results is that the primary problem in *recA* cells is not in getting the replication complex to pass template strand lesions but in the subsequent processing of those structures that are present as a consequence of the replication fork having already passed a significant number of lesions.

### Newly Synthesized DNA in UV-Irradiated Cells Is Associated with Single-Stranded Regions

In order to search for and quantitate single-stranded regions associated with newly replicated DNA, a column method that separated DNA molecules as a function of the degree of single strandedness was developed. This method was used to estimate that the DNA synthesized shortly after UV irradiation had single-stranded regions that corresponded roughly to the size of an Okazaki fragment for each lesion in the template strand (26). In addition, these data also showed that the interruptions in the newly synthesized strands observed previously in alkaline sucrose gradients by Rupp and Howard-Flanders (72) were due to gaps rather than to cryptic lesions that led to alkaline-induced strand breaks.

The data from Iyer and Rupp (26) can also be used to estimate the amount of single-stranded DNA at each replication fork, an interesting calculation that was not done in the original paper. Iyer and Rupp (26) estimated that the DNA synthesized in the first 10 min after UV irradiation was 3.0% single stranded with a UV dose that reduced the DNA synthesis level to 25% of that of the control (72). The normal *E. coli* replication fork moves at about 1,000 bp/s at 37°C. From these numbers, it follows that about 9,000 bases of single-stranded DNA are generated at each replicating fork in the first 10 min after irradiation. This should not be considered a precise measurement, because the column method is rather crude, and elution of the experimental sample from the column was more heterogeneous than elution of the standard. However, the experimental design used in this procedure systematically underestimates the amount of single-stranded DNA, because the label is in the newly synthesized strand while the single-stranded bases are expected in the template strand. Intentional shearing to produce a size comparable to that of the standard certainly broke off any long single-stranded tails, which would not then be scored by this method, because the single-stranded fragments would no longer be associated with the label in the newly synthesized strands. (Johnson and McNeill [29] in fact observed very long single-stranded regions in UV-irradiated cells.) Thus, the actual amount of single-stranded DNA might well be considerably greater than the 9,000 nt calculated above.

How does this amount of single-stranded DNA relate to the amount of the *E. coli* DNA-binding protein SSB present in a cell? The generally accepted view is that the amount of single-stranded DNA in a cell under normal conditions is quite small and that the amount of SSB exceeds this amount by a considerable margin, so that any single-stranded DNA is rapidly coated by SSB. According to Kornberg and Baker (34), there are about 270 SSB monomers per replication fork, which are sufficient to cover about 2,000 to 4,000 nt of single-stranded DNA. In their review, Chase and Williams (12) stated that the SSB in a cell covers an average of about 1,400 nt per replication fork. Since

the level of SSB is apparently not increased significantly after UV irradiation (63, 121), this calculation shows that in a UV-irradiated cell, the amount of single-stranded DNA generated at a replication fork soon exceeds the amount that can be coated by SSB. This exposed single-stranded DNA is highly significant in several respects. It will certainly be a site for binding of RecA protein, leading to strand exchange and activation of SOS responses through cleavage of the LexA repressor (87). Another highly significant property of single-stranded DNA lacking SSB is that it allows several modes of primer formation by the DnaG primase to occur that are prevented when single-stranded DNA is coated with SSB (34). Such primer formation may play a role in the cycling of polymerase III holoenzyme to new primed sites beyond a blocking lesion on the leading strand template, as discussed in more detail below.

## What Is the Effect of Polymerase-Blocking Lesions at the Replicating Fork in UV-Irradiated Cells?

In attempting to understand events in irradiated cells, we briefly consider several features of replication in unirradiated cells. (This topic is considered in detail in chapter 50 in this volume.)

**Coupling of DnaB Helicase and Polymerase III Holoenzyme at the Replication Fork.** A key element of current DNA replication models is that a replication fork moves in one direction and uses DNA polymerases that have a unique polarity of synthesis (5′ to 3′ for the new strand) to achieve duplication of two DNA strands with differing polarities. Though one strand (the leading strand) can be synthesized continuously in the same direction as fork movement, the other strand (the lagging strand) is synthesized discontinuously in many segments (Okazaki fragments), with the individual pieces being generated in a direction opposite to the direction of overall movement of the fork. Mechanistically, the cell presumably accomplishes this with a replicative polymerase III holoenzyme that acts processively while remaining attached to one side of the fork to synthesize the leading continuous strand, while the discontinuous synthesis of the lagging strand is accomplished by a polymerase III holoenzyme that must repeatedly cycle on and off its template strand. A dimeric polymerase III holoenzyme might be able to accomplish synthesis of both the leading and the lagging strands concurrently (11, 34, 48, 49).

It is clear that a replication fork is much more complex than just a polymerase copying template strands. The DnaB helicase is a key player in the formation and propagation of replication forks (3, 4, 38, 47, 70, 125). The insertion of the DnaB helicase in a double-stranded DNA at the replication origin is a keystone event in forming a replication fork to start a round of replication. Once DnaB is inserted into the DNA, it apparently remains at the front of the replication fork until the replication of that replicon is finished. Mok and Marians (55) developed an in vitro replication system for rolling-circle molecules and demonstrated a very high rate of fork movement that required polymerase III holoenzyme plus helicase activity from either DnaB or the preprimosomal proteins (a primosome without DnaG primase). In this system, the helicase and polymerase III holoenzyme were extremely processive (>50,000 nt), with a fork moving at a rate similar to the in vivo rate. In this coupled system, the fork was moving at a rate faster than the helicase by itself is known to separate strands. (Although this result might suggest specific protein-protein interactions between DnaB and the polymerase III holoenzyme, the fact that the polymerase III holoenzyme's rate of polymerization on a primed single-stranded DNA template exceeds the rate at which the DnaB helicase can move through duplex DNA to separate the strands could be sufficient to maintain intimate contact between DnaB and the holoenzyme at the replicating fork and perhaps to even "push" the helicase so that it separates strands faster in the coupled situation than when acting by itself.) Under normal conditions, in which leading-strand elongation is coupled with helicase movement at the fork, the template for leading-strand extension is copied so efficiently that no single-stranded DNA between DnaB and polymerase III interacts with SSB, either because this DNA is too short or because it is protected by proteins in the replication complex or both.

**Effect of DNA Lesions on Activity of DnaB Helicase.** Oh and Grossman (62) reported that DnaB helicase activity is little affected by UV irradiation of the substrate. As an extension of this observation, a DnaB helicase substrate was constructed to mimic the situation in which a bulky lesion is present in the template for leading-strand synthesis. With this construct, there was no inhibition of the DnaB helicase activity (Fig. 7). Extrapolating these results to a replicating fork, I suggest that the DnaB helicase in a replication complex at a fork will not be blocked by lesions such as pyrimidine dimers in the duplex DNA and can continue to move along and separate the two strands even though they contain lesions that block polymerase III holoenzyme.

**Cycling of Polymerase III Holoenzyme.** During replication of *E. coli*, the polymerase III holoenzyme must cycle to the next Okazaki fragment every second or two (10, 34, 110). In contrast, although the purified enzyme rapidly and processively replicates a primed single-stranded-DNA circle, several minutes are required to cycle to the next primed single-stranded-DNA circle. O'Donnell and his colleagues (61, 109, 110) studied this process and demonstrated that the cycling time can be reduced to 10 s when the primed acceptor single-stranded DNA has an appropriate preinitiation complex that comprises a subassembly of the polymerase III holoenzyme. The preinitiation complex is a "protein clamp on primed ssDNA formed by the accessory protein β and the five-protein γ complex ($\gamma\delta\delta'\chi\psi$)," and it was concluded that the γ complex acts catalytically in forming a β clamp on the primed template (61, 109, 110). The cycling takes place only when a fragment has been completed, and cycling is thought to proceed through a bimolecular reaction in which part of the holoenzyme is transferred directly from the completed molecule to the subassembly on the acceptor molecule to form an active replication complex on the acceptor. This mode of facilitated transfer without dissociation from the completed fragment is of obvious value for repetitive synthesis of the lagging-strand Okazaki fragments. We expect that these cycling reactions play a central role in the processing of the newly synthesized fragments in UV-damaged cells.

In an earlier study of UV-irradiated DNA, Shavitt and Livneh (100, 101) studied the cycling of polymerase III holoenzyme. Their results showed that cycling from one molecule to another is slowed by UV irradiation but that increasing the amount of the β subunit (known to be part of the preinitiation complex) decreases the cycling time, presumably by facilitating the dissociation of the polymerase III holoenzyme from locations where it was stalled at a photoproduct. In these experiments, cycling times were in minutes rather than seconds, presumably because

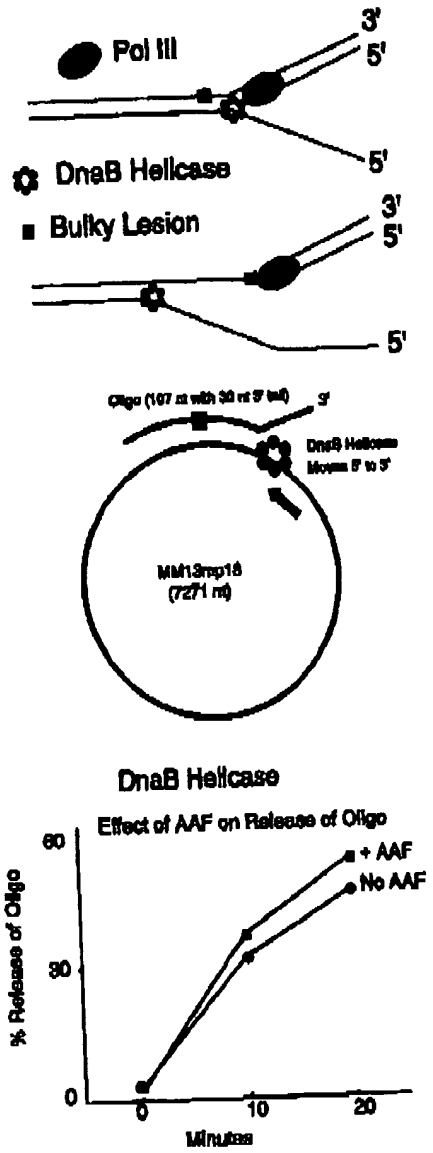


FIGURE 7 Uncoupling of DnaB helicase and polymerase III at a lesion in the DNA strand coding for synthesis of the continuous strand. (Top) Model for replication fork with polymerase III and DnaB helicase at the replicating fork approaching a bulky lesion. Polymerase III is blocked at the lesion, but the helicase continues past the lesion. (Center) Model substrate for DnaB constructed with the AAF bulky lesion to mimic the structure given above but without polymerase III. (Bottom) The results show that AAF on the oligonucleotide does not prevent release of the oligonucleotide. This finding is consistent with the suggestion that DnaB helicase at the replicating fork might proceed past bulky lesions in the strand that serves as template for synthesis of the continuous strand.

these studies did not generate in advance the preformed preinitiation complexes on the acceptor single-stranded DNA molecules that are necessary for the very fast cycling observed by Studwell et al. (110).

**Lagging-Strand Synthesis.** During the discontinuous synthesis of the lagging strand on a normal undamaged template, the DnaG primase interacts with DnaB helicase at the fork to synthesize primers for the lagging strand. These primed sites are then used by polymerase III holoenzyme to generate the lagging-strand segments that are subsequently joined together to form an intact lagging strand. The polymerase III holoenzyme cycles efficiently from one primed site to another during this process. Although DnaG primase interacts with DnaB helicase at the fork, this interaction seems to be transient, since the reaction of DnaG primase is apparently distributive rather than processive (49).

**Lesion on Lagging-Strand Template at the Replication Fork.** A lesion on the lagging-strand template at the replication fork is similar to that occurring during synthesis of the lagging strand on a normal template. The priming by DnaG can occur normally to generate a primed site for polymerase III holoenzyme. Instead of completing a whole segment, the polymerase III will presumably stop at the lesion, but from here it can be recycled to the next primed site on the lagging-strand template. This gap, which extends from the lesion to the 5′ end of the next fragment, is single stranded and presumably accounts for a significant amount of the single-stranded DNA generated in UV-irradiated cells.

**Lesion on Leading-Strand Template at the Replication Fork.** When a replication complex runs into a lesion on the leading-strand template, the resulting structure will be quite different from the normal undamaged case. The DnaB helicase will presumably continue separating strands and will proceed right on past the lesion. However, the situation with the polymerase III holoenzyme will differ, in that the holoenzyme will stall when it reaches the lesion in the template strand, thus uncoupling leading-strand synthesis from the helicase movement at the fork. This abnormal situation with the polymerase III holoenzyme stalled at the lesion while the DnaB helicase at the fork continues separating the two strands will generate a stretch of single-stranded DNA extending from the lesion on the leading-strand template where the polymerase III holoenzyme is stalled to the slowly advancing DnaB helicase at the fork (Fig. 7 and 8).

**Uncoupling of DnaB Helicase and Polymerase III Holoenzyme: Priming and Polymerase Cycling on Leading Strand.** The continuous synthesis of the leading strand is a generally accepted central tenet of current replication fork models. Thus, the requirement in our interpretation that the leading strand must frequently be restarted seems bizarre. However, in considering known reactions of relevant enzymes, this possibility becomes credible. The first step in the process must be a priming event. The first and most efficient possibility, if it occurs, is a DnaB-DnaG priming event at the fork that is analogous to the priming of the lagging-strand fragments. In the coupled situation, all the priming events are on the lagging-strand template. In the uncoupled situation, frequent priming events do occur, but it has not been determined whether these are exclusively on the lagging-strand template or whether they also take place on the leading-strand template (3, 4). In the coupled situation, the exclusive synthesis of primers on the lagging-strand template may be explained simply by differences in the accessibility of the two strands when they are first separated by the advancing DnaB helicase. The leading-strand template is copied efficiently by polymerase III holoenzyme before it is accessible to SSB, so it is not surprising that the DnaG primase could not use it for primer formation. However, when elongation of the leading strand is interrupted by a photoproduct at which polymerase III is

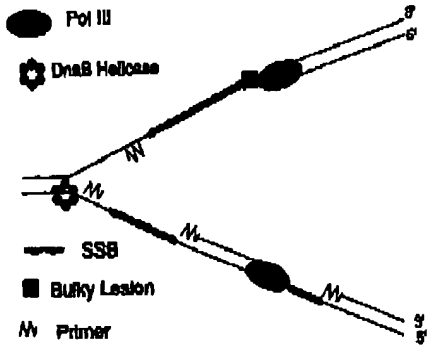


FIGURE 8 Model for restart of the leading strand when a replication fork reaches a polymerase-blocking lesion.

stopped, the separated strands formed by the uncoupled advancing DnaB helicase might well become a substrate for DnaG primase priming on the template for the leading strand as well as for the lagging strand. This is only one of the hypothetical possibilities, and other priming reactions might come into play during uncoupled conditions either instead of or in addition to direct interaction of DnaG with DnaB helicase at the fork. One possibility that is normally considered to be physiologically irrelevant, the general priming reaction (34), should be mentioned, because as pointed out in the calculation in a previous section, the quantity of single-stranded DNA generated soon after UV irradiation might be greater than can be coated by SSB. Thus, while the general priming reaction may not occur in unirradiated cells, it may become significant after UV irradiation under exactly those conditions in which the leading strand must be restarted.

**Primosomes with PriA, PriB, and PriC May Be Involved in Restarts.** PriA, PriB, and PriC are not essential for normal replication, since strains with mutants in the single genes are viable, although somewhat sick (39, 60, 133, 134). However, double mutants with *recA* are not viable. A potential role for these gene products is to facilitate the movement of the replication fork past certain problem areas in the chromosome that might be barriers for replication, such as unexcised bulky lesions. Perhaps bulky lesions are a more serious obstacle for the DnaB helicase in the template for lagging-strand synthesis than in the leading-strand template, since the polarity of DnaB predicts that the lagging-strand template will be tracked. The dual polarity of the primosome with PriABC might provide a backup helicase activity for proceeding beyond such barriers (2, 40, 133).

Once a primer is formed, the polymerase III holoenzyme must bind to and extend the primer to restart the leading strand. When this stage is reached, the extension will be rapid, the polymerase III holoenzyme will catch up with the more slowly moving uncoupled DnaB helicase, a coupled replication complex will be reestablished, and this complex will then move at the normal rapid velocity (1 kbp/s) expected for normal replication fork movement.

This mechanism requires cycling of the polymerase III holoenzyme on the leading strand at lesions as well as on the lagging strand. In our original study, we estimated that the average delay per dimer was about 10 s (72). If only the lesions in the leading strand cause a delay, then this rate is equivalent to 20 s per lesion. It was estimated that during normal replication, the lagging strand is primed every 1 to 2 s. In the experiments of Studwell et al. (110), cycling with model substrates was fast and was comparable to the calculated delay at a pyrimidine dimer.

Maintenance of DnaB helicase at a fork may be sufficient to retain a replication fork if polymerase III holoenzyme can recycle past lesions on the leading-strand template, because the polymerase will soon catch up with the slowed helicase when synthesis resumes at a primed site on the leading-strand template. As pointed out before, the limiting factor in forming replication forks is the installation of the DnaB helicase at replication origins, not the availability of the polymerase III holoenzyme.

**Translesion Synthesis.** It is well documented that UV-produced lesions such as pyrimidine dimers are effective blocks to DNA polymerases in purified systems and inside cells (15). It is also now clear that direct bypass does occur at low frequencies with purified polymerase I or polymerase III (67, 115). Results with a thymine-thymine dimer incorporated at a specific site in a vector are particularly clear (5). When this single-stranded circle was used to transfect an excision-defective host, survival was less than 0.5% of that of the control, demonstrating that bypass was very rare. However, when the host was UV irradiated to induce SOS functions, survival rose to 25 to 30%, showing that in vivo bypass can occur with a high frequency at a particular lesion. This is a very specialized situation, and the results may have only limited applicability to a lesion in a double-stranded molecule. In the single-stranded circle, the alternatives are limited when a polymerase reaches a blocking lesion; it (or another polymerase) can keep trying to bypass the lesion until the template molecule is inactivated (for example, by nuclease attack). In a double-stranded molecule, competing reactions such as recombinational exchange or polymerase cycling to another site may decrease the probability of bypass at a particular site except in cases such as overlapping daughter strand gaps, where recombinational exchanges do not provide feasible alternatives.

What is the contribution of direct translesion synthesis after UV irradiation? UV mutagenesis depends on the *umuC* and *umuD* gene products, presumably as a result of mutagenic translesion synthesis. How frequently does this synthesis actually occur, and what effect does it have on survival of UV-irradiated cells? Experiments were done to determine the effect of constructing a double mutant defective in both *umuC* and *uvrA*. Walker and Dobson (126) did not observe any increased sensitivity due to the *umuC* mutation in either wild-type or *uvrA6* excision-defective strains. Thus, under conditions in which the recombination systems are active, any enhanced survival promoted by translesion synthesis dependent on UmuC is minimal and can represent only a small fraction of those events that occur when a replication fork reaches a blocking lesion. While the UmuCD-dependent bypass of lesions is quantitatively dominant for UV mutagenesis, these error-producing events arise from a very small fraction of the total repair events.

**Inhibition of DNA Synthesis by UV Irradiation: Induced Replisome Reactivation and Replication Restart.** Transient inhibition of DNA synthesis in UV-irradiated *E. coli* was studied by Khidir et al. (30) and Witkin et al. (128). This phenomenon has been termed Irr (induced replisome reactivation) or replication restart (15, 30, 128). These studies, which show a requirement for RecA and a second additional factor, are complicated by the fact that most of the experiments were done in an excision-proficient

background that allowed rapid removal of lesions after irradiation. However, it is clear from the data in both papers that after irradiation, although DNA synthesis was markedly inhibited, substantial residual synthesis continued even when the Irr or replication restart did not occur. The interpretations of the various authors are that lesions bring the replisome to a complete halt and that the replisome must be "reactivated" or "restarted" to allow DNA synthesis to continue. However, if the interpretation offered here is correct, i.e., that blocking lesions cause uncoupling of polymerase III and DnaB activity of the replication complex rather than a complete block, then the interpretation of these data will be rather different.

Thus, if a replisome is not blocked at a lesion, it does not need reactivation or a restart to pass the lesion. Inhibition initially occurs because template strand lesions uncouple the helicase and the polymerase at the replication fork, causing the overall movement of the fork to slow down but not stop. In this model, inhibition might persist if the passage of the replication complex generates structures that might be resistant to the normal modes of repair and thus accumulate to provide a sink that competes with the fork for components that might be limiting, such as the polymerase III holoenzyme. In this situation, translesion synthesis might well be the mechanism to relieve the observed inhibition, but this translesion synthesis would not be required to occur directly at the fork to get it restarted but would occur at structures generated by the previous passing of the DnaB helicase component of the replication complex. The third role of RecA (67, 112) in addition to cleavage of LexA and UmuD, could be a protective effect of its binding to single-stranded DNA. This effect might be particularly critical, since the calculations given above indicate that the level of SSB may be insufficient for the amount of single-stranded DNA generated soon after UV irradiation.

In this model, DNA synthesis can be inhibited in at least two ways by uncoupling DnaB helicase activity from polymerase III chain elongation of the leading strand at the replication fork. First, when DnaB helicase is uncoupled, it separates strands much more slowly than when it is coupled with polymerase III in the normal fork configuration. In the second, a somewhat indirect way, unrepaired pieces left in the wake of the advancing uncoupled fork could be competitive sinks for polymerase III holoenzyme, particularly if they retain either the entire polymerase III enzyme or a subassembly of the holoenzyme, as might occur in the facilitated bimolecular transfer of the polymerase III core to a second preprimed location. The accumulation of significant quantities of these unrepaired termini with preinitiation complexes remaining attached could compete with the replication fork for the limited number of polymerase III holoenzyme molecules in the cell. If these locations are at closely spaced lesions or overlapping daughter strand gaps, SOS-mediated translesion synthesis requiring UmuCD′ might be required to remove inhibitory unrepaired competitive termini (92, 93). Sommer et al. (107, 108) suggested that the UmuCD′ proteins compete with RecA and switch from homologous recombination to SOS mutagenesis and that this switch occurs slowly because the induction of the UmuCD proteins is delayed.

## Summary

Although many steps and many polypeptides are required for replication, the single most important element that is required for beginning and maintaining a round of replication is the insertion of the DnaB helicase at the replication origin and its continued ordered processive advance through the replicon. Its entry into the DNA precedes entry of the polymerase III holoenzyme, and its location at the front of the replication fork separates the strands for copying by polymerase III. It is clear from the work of Baker et al. (3) that the DnaB helicase can separate strands for long distances on its own and that priming can occur so that the subsequent addition of polymerase III holoenzyme results in rapid copying of the exposed regions, presumably until polymerase III either catches up with the more slowly moving DnaB helicase or comes to another duplex region. For a replication complex in an unirradiated cell, synthesis of the leading strand will be synchronous with advancement of the DnaB helicase, because the rate of chain elongation is greater than the movement of DnaB helicase alone. (It is not clear whether DnaB's higher velocity under coupled conditions is due to specific protein-protein interaction between polymerase III and DnaB or whether the rapid polymerization on the leading-strand template exposed by DnaB is sufficient to bring the polymerase III holoenzyme into direct contact with DnaB and simply push the DnaB helicase faster without any specific interactions between the proteins.) Coordination with lagging-strand synthesis is accomplished in two ways. First, the lagging-strand primers require the interaction of DnaG primase with DnaB helicase. Second, the polymerase III holoenzyme has a dimeric structure with two core enzyme units, so that polymerization on the leading and lagging strands can take place simultaneously with the same dimeric polymerase molecule. It has thus been proposed that while one of the core units of the dimeric polymerase III continuously extends the leading strand, the second part of the dimeric molecule continuously recycles to generate the discontinuous Okazaki pieces of the lagging strand.

What effect does DNA damage have on this process? The main points are that the replisome or replication fork may not always behave like a monolithic unit, and that the assumption that the entire replication fork stops at a lesion just because one component of the replication complex, the polymerase III holoenzyme, stops there is probably an inaccurate oversimplification. The continued progress and integrity of a replicating fork may well be determined by what happens to the DnaB helicase rather than by what happens to a particular polymerase III holoenzyme molecule. Although polymerase III can easily start elongating chains from an appropriate primer at any location, the correct insertion of DnaB helicase to create a replication fork is a highly specialized reaction that occurs efficiently only at replication origins.

## CONCLUSION

In this chapter are discussed the variety of specific and nonspecific mechanisms that bacteria have developed to meet the challenges of the many alterations that are introduced into DNA.

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